Isoterchebulin and 4,6-*O*-Isoterchebuloyl-D-glucose, Novel Hydrolyzable Tannins from *Terminalia macroptera*

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Two new hydrolyzable tannins, isoterchebulin (**1**) and 4,6-*O*-isoterchebuloyl-D-glucose (**2**), together with six known tannins, **3**−**8**, were isolated from the bark of *Terminalia macroptera*. Their structures were elucidated by extensive 1D and 2D NMR studies, MS, and chemical transformations. Biological activities of all compounds were evaluated against the snail *Biomphalaria glabrata*, the bacteria *Bacillus subtilis* and *Pseudomonas fluorescens*, the nematode *Caenorhabditis elegans*, and four cancer cell lines (Hep G2, MCF-7/S, MDA-MB-231, and 5637 cells). All compounds except **3** showed antimicrobial activities against *B. subtilis* (MIC 8−64 μg/mL), whereas only **1** was active against *C. elegans* (100 μg/mL) and *B. glabrata* (LC_{100} = 60 μg/mL). **3** and **8** were toxic against 5637 cells with LC_{50} = 84.66 and 41.40 μM, respectively.

Terminalia macroptera Guill. et Perr. (Combretaceae) is a tree widely distributed in Africa. In traditional African folk medicine different parts of the plant, including leaves, shoots, seeds, fruit galls, roots, and bark, are used for treating various diseases and ailments.[1] Leaf and bark extracts showed strong antimicrobial activity toward the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*,[2] while a root extract was active against several strains of *Neisseria gonorrheae*.[3] An ethanol extract of the bark exhibited a minimum lethal dose of 250 mg/kg in rats.[4] Previous phytochemical investigations of the flowers led to the isolation of several *C*- and *O*-glycosyl flavones.[5−7] Chlorogenic acid, quercetin, isoorientin, the ellagitannins chebulagic acid, chebulinic acid, punicalagin, and terflavin A, gallic acid, and ellagic acid were isolated from the leaves,[8,9] while different methylated ellagic acid derivatives and the triterpenoid terminolic acid were obtained from the heartwood.[10]

We reported previously on the isolation of two galloylated pentacyclic triterpenoids and a phenolic glucoside gallate from the bark of *T. macroptera*.[11,12] In the present paper we describe the isolation and structure elucidation of two novel hydrolyzable tannins (**1**, **2**) having a new tetraphenylic acid moiety (isoterchebulic acid), together with six known tannins (**3**−**8**, Supporting Information). We also report on the biological properties of these compounds including molluscicidal, antibacterial, nematicidal, and cytotoxic activities.

Results and Discussion

An EtOAc extract of the dried bark was chromatographed on Sephadex LH-20 followed by HPLC separation on RP18 to give the new tannin isoterchebulin (**1**). The MeOH extract was dissolved in H_2O and extracted with *n*-BuOH. The *n*-BuOH layer was concentrated in vacuo and partitioned between $CHCl_3$, MeOH, and H_2O. The aqueous layer was separated by column chromatography on polyamide SC60 and Sephadex LH-20 as well as HPLC using RP18 or CN-Phase to afford 4,6-*O*-(*S*)-isoterchebuloyl-D-glucose (**2**) together with the six known tannins: 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**3**),[13] punicalagin (**4**),[13,14] terflavin A (**5**) and B (**6**),[15] 2-*O*-galloyl-punicalin (**7**),[16] and punicacortein C (**8**).[17] The structures of the known tannins were elucidated on the basis of 1D and 2D NMR (^{1}H, ^{13}C, DQF-COSY, HSQC, and HMQC), ESIMS, and optical rotation $[\alpha]_D$. The experimental data for **3**−**8** were in agreement with values obtained from the literature.

Isoterchebulin (**1**) was isolated as a tan amorphous powder. Its molecular formula of $C_{48}H_{28}O_{30}$ was established by HRFABMS. The ^{1}H NMR spectrum of **1** showed duplicated signals in a ratio of 4:3 for the sugar and phenolic moieties, suggesting that the anomeric center of the sugar moiety was not acylated. The ^{13}C NMR spectrum displayed 12 signals due to sugar carbons including two anomeric carbons at δ 94.2 (C-1$_\beta$) and 90.8 (C-1$_\alpha$), which confirmed that **1** existed as an equilibrium mixture of α and β forms in solution (acetone-d_6: D_2O = 9:1). The connectivities of the seven sugar protons in both anomeric moieties were determined by COSY, and their directly bonded carbons were assigned by GHSQC (Table 1). ^{1}H−^{13}C long-range correlations (GHMQC) between the anomeric protons H-1 (α: δ 5.34; β: δ 5.01) and C-5 (α: δ 70.09; β: δ 74.51) along with H-5 (α: δ 4.12; β: δ 3.89) and C-1 (α: δ 90.81; β: δ 94.20) in each sugar unit indicated a pyranose ring with an ether linkage between C-1 and C-5. The observed vicinal coupling constants of $J \approx 9$ Hz between the trans diaxial oxymethine protons H-2 and H-3, H-3 and H-4, and H-4 and H-5 (each form) together with J = 8.08 Hz for H-1$_\beta$ and H-2$_\beta$ and J = 3.49 Hz for H-1$_\alpha$ and H-2$_\alpha$ established a glucopyranosyl moiety with $^4C_1$ conformation for both sugars in the case of a D configurated pyranose. Furthermore, deshielding of more than 1 ppm of the protons H-2, H-3, H-4, and H-6 implied a tetra-acylated glucose in **1** (Table 1). Complete acid hydrolysis liberated the sugar moiety, which was identified as D-glucose by TLC and optical rotation ($[\alpha]^{25}_D$ = +59) in comparison with an authentic sample.

A total of 10 aromatic protons for both forms resonating as sharp singlets each were found in the chemical shift

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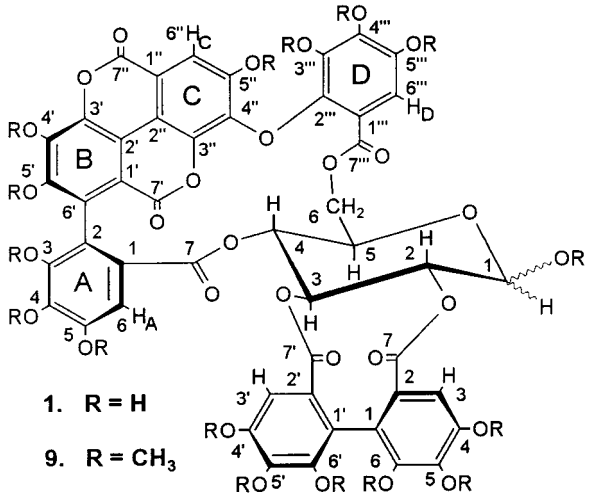
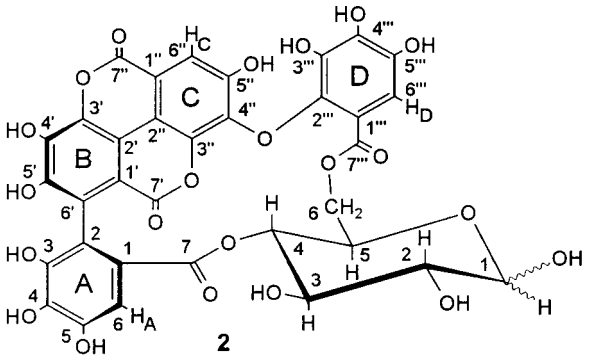
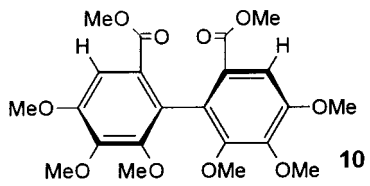
Table 1. ^{1}H NMR and ^{13}C NMR Spectral Data of the α- and β-D-Glucose Residues of Compounds **1** and **2** (acetone-d_6/D_2O = 9:1)

	1		**2**	
pos	δ,[a] mult; *J* [Hz]	^{13}C[a]	δ,[a] mult; *J* [Hz]	^{13}C[a]
1α	5.34, d; 3.5	90.81	5.03, d; 3.6	92.86
2α	4.93, dd; 9.4, 3.4	74.64	3.37, dd; 9.8, 3.4	73.04
3α	5.62, t; 9.6	75.05	4.02, t; 9.7	71.51
4α	4.78, t; 9.8	69.61	4.35, t; 9.5	73.16
5α	4.12, bt; 9.2	70.09	4.03, bt; 9.4	69.94
6α	4.46, dd; 12.0, 8.6 3.14, bd; 11.9	64.59	4.31, dd; 11.7, 8.7 3.07, bd; 11.8	64.95
1β	5.01, d; 8.1	94.20	4.47, d; 7.8	97.18
2β	4.73, dd; 9.6, 8.1	77.11	3.19, dd; 9.4, 7.9	75.55
3β	5.34, t; 9.6	77.47	3.74, t; 9.2	74.75
4β	4.75, t; 9.8	69.37	4.36, t; 9.5	72.83
5β	3.89, bt; 9.1	74.51	3.58, bt; 9.3	74.26
6β	4.42, dd; 12.0, 8.3 3.14, bd; 11.9	64.44	4.29, dd; 12.0, 7.4 3.08, bd; 11.8	64.95

[a] ^{1}H NMR (500 MHz), ^{13}C NMR 125.72 MHz (compound **1**) and 75.42 MHz (compound **2**). δ in ppm. Observed coupling constants were not averaged. Observed triplet signal patterns (t, bt) in the ^{1}H NMR spectra correspond to pseudotriplet signals. Assignments based on COSY, DQFCOSY, GHSQC, and HMBC spectra.







region of δ 6.35−7.57, indicating the presence of five pentasubstituted aromatic rings. The low-field shift of the proton at δ 7.56/7.57 (α/β form) was attributable to a depside-type linkage between two aromatic rings, which is known in the case of hydrolyzable tannins, e.g., terflavin A, B[15] and terchebulin.[18] The chemical shift values of eight

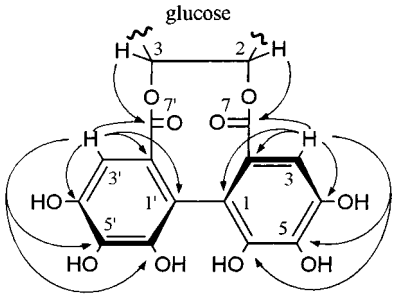


Figure 1. HHDP moiety attached to O-2 and O-3 of the central glucose core of **1**. Arrows exhibit ^{1}H−^{13}C long-range correlations.

carbons in the region of δ 166.6−169.8 and four carbons at δ 157.7−160.0 in the ^{13}C NMR spectrum suggested the presence of four carbonyl ester functions and two aromatic δ-lactone rings for each form similar to punicalagin[13,14] and terchebulin.[18] From the remaining 72 aromatic carbons only 63 could be seen in the ^{13}C NMR spectrum, due to overlapping. Ten carbons in the region δ 106−114 were identified as hydrogen-bearing aromatic carbons by GHSQC. The assignment of the remaining closely resonating quaternary carbons between δ 105 and 150 was achieved by a series of highly optimized GHMQC experiments using two different concentrations (60 and 300 mg/700 μL, respectively), 4096 increments in the F1-direction for the maximum resolution of the correlating signals, and different $^nJ_{C-H}$ optimized for 1, 2, 3, 5, and 8 Hz. Inspection of the HMBC spectra revealed that a $^nJ_{C-H}$ optimization of 5 and 8 Hz contained the most information about the connectivities inside the sugar moiety. However, for the determination of the polyphenolic substructures, optimizations for small long-range couplings on the order of 1 and 2 Hz were necessary to link as many quaternary carbons as possible (^2J, ^3J, and ^4J) to the few remaining aromatic protons. For the suggested structure only one hydrogen per aromatic ring was proposed.

The hexahydroxydiphenoyl (HHDP) substructure (Figure 1) was represented in the ^{1}H NMR spectrum by the aromatic protons H-3 at δ 6.610(α)/6.615(β) and H-3′ at δ 6.35(α)/6.37(β). Carbons C-3 and C-3′ were identified by GHSQC at δ 107.5 and δ 106.93/106.90, respectively. Each of the protons H-3 and H-3′ of the HHDP moiety exhibited ^{1}H−^{13}C long-range correlations to an ester carbonyl carbon (C-7: δ 169.05/168.95; C-7′: δ 169.82), two carbons corresponding to aromatic C−C bonds (C-1, C-1′: δ 114.59, 114.49, 114.47 and 114.46; C-2: δ 126.32/126.11; C-2′: δ 126.33) and to three oxygen-bearing aromatic carbons (C-4, C-4′: δ 145.16, 145.15, 145.26, and 145.23; C-5: δ 136.24/136.15; C-5′: δ 136.27; C-6, C-6′: δ 144.41, 144.47, and 144.35). In agreement with the ^{13}C NMR data obtained for the HHDP moiety in compound **3** these results established an HHDP substructure in **1**. ^{1}H−^{13}C long-range correlations between the low-field glucose protons H-2 (α: δ 4.93; β: δ 4.73) and the HHDP carbonyl carbon C-7 as well as H-3 (α: δ 5.62; β: δ 5.34) and C-7′ indicated that the HHDP unit is attached to O-2 and O-3 of the central glucose core by ester linkages. These findings were confirmed by partial hydrolysis, which besides the starting material produced ellagic acid and a hydrolysate identical to the new 4,6-*O*-(*S*)-isoterchebuloyl-D-glucose (**2**). Ellagic acid[19] was identified by EIMS and ^{1}H and ^{13}C NMR.

The structure of the novel tetraphenylic acid moiety (isoterchebulic acid) was elucidated as outlined in Figure 2: ^{1}H−^{13}C long-range correlations between the ester carbonyl carbon C-7 (δ 167.45/167.38) and the low-field glucose proton H-4 (α: δ 4.78; β: δ 4.75) along with the aromatic

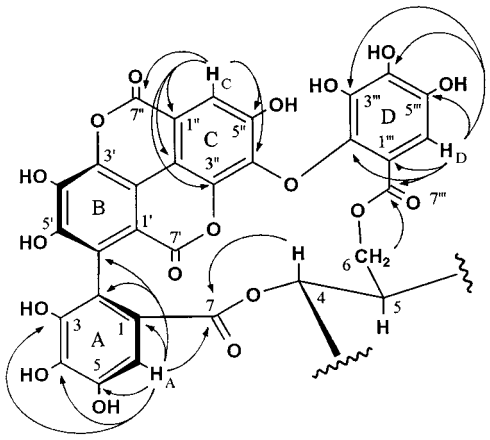


Figure 2. Isoterchebuloyl unit attached to O-4 and O-6 of the central glucose core of **1**. Arrows exhibit $^1H-^{13}C$ long-range correlations.

proton H_A-6 (δ 6.79(α)/6.76(β); C-6: δ 109.59/109.51 by GHSQC) indicated that the aromatic ring A is linked to O-4 of the glucose core. The remaining five ring A carbons were identified by GHMQC at δ 145.17/144.59, 144.59, 137.72/137.63, 123.64/123.40, and 116.50/116.35. Comparison of these values with those of one phenolic ring of the HHDP moiety suggested an esterified gallic acid derivative with a similar attachment to another aromatic ring as in the HHDP unit.

The aromatic ring system D was represented in the ^1H NMR spectrum by the proton H_D-6‴ at δ 6.535(α)/6.530(β) (C-6‴: δ 107.55 by GHSQC). In the HMBC spectrum H_D-6‴ was correlated with an ester carbonyl carbon (C-7‴: δ 166.73/166.67) and one carbon signal at δ 114.92/114.77, which was assigned to the ester carbonyl bearing carbon and, in contrast to ring A, to four oxygen-bearing aromatic carbons at δ 142.45, 138.77/138.60, 138.06/137.99, and 138.97/138.94. The 2,3,4,5-tetrahydroxy substitution pattern of the tetrahydroxy benzoic acid moiety of ring D was deduced by comparison of the observed ^{13}C NMR data with computer-generated carbon spectra of the three possible constitution isomers of tetrahydroxy benzoic acid.

The low-field-shifted aromatic proton H_C-6″ (δ 7.56(α)/7.57(β); C-6″: δ 112.55/112.52 by GHSQC) implied the occurrence of a depside-like linkage of ring C. In the HMBC spectra this proton correlated with an upfield-shifted carbonyl carbon (C-7″) at δ 160.00, which was assigned to an aromatic δ-lactone ring. $^1H-^{13}C$ long-range correlations between H_C-6″ and the five carbons at δ 150.97/150.91, 141.70/141.64, 139.03/139.00, 112.83, and 113.61 established an additional gallic acid derivative for ring C. The remaining 14 carbons (two of them overlapped) in the ^{13}C NMR spectrum showing no $^1H-^{13}C$ long-range correlations as in the rings A, C, and D were attributed to another 2′,6′-substituted gallic acid unit (aromatic ring B). Comparison of the ^{13}C NMR data of rings B and C with those for punicalagin[12,13] and terchebulin[17] suggested that rings B and C form an ellagic acid substructure located between rings A and D. The orientation of the ellagic acid derivative between rings A and D (Figure 2) was established by the $^4J_{C-H}$ long-range coupling from the proton H_A-6 to the quaternary carbon C-6′ of ring B at δ 124.05/124.02, which can only be observed in the case of a C−C linkage between C-2 of ring A and C-6′ of ring B.

Considering the 13 carbons of the isoterchebuloyl moiety that were assigned as phenolic carbons including the two associated with the aromatic δ-lactone rings, a molecular weight of 1102 mu should be expected for **1**. The difference of 18 mu from the observed molecular mass ($[M - H]^-$ at *m/z* 1083 in FAB as well as ESIMS) could be explained by an ether linkage between two phenolic hydroxy groups. However, the position of the ether linkage between ring C and D was not assignable by direct NMR measurements. Therefore, **1** was permethylated with DMS and K_2CO_3 in dry acetone to yield a mixture of the α and β forms of the hexadecamethyl ether **9**, which was identified by ^1H NMR and ESIMS ($[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ ions at *m/z* 1309, 1331, 1347, respectively). A mass difference of 224 mu equivalent to 16 introduced methyl groups and the occurrence of 16 methoxy groups in the ^1H NMR spectrum confirmed the presence of 16 hydroxy groups in **1**. Methanolysis of **9** with sodium methoxide in dry methanol afforded dimethyl-(*S*)-hexamethoxydiphenoate (**10**), which was identified by ^1H NMR, EIMS, optical rotation, and CD together with the nonamethylisoterchebulic acid dimethyl ester (**11**).

The ESIMS spectrum of **11** displayed its $[M + Na]^+$ ion at *m/z* 815. The ^1H NMR spectrum exhibited three aromatic protons at δ 7.46 (H_A-6), 7.74 (H_C-6″), and 7.21 (H_D-6‴) and 11 sharp singlets (3H each) in the chemical shift region δ 3.5−4.2 which were assigned to 11 methoxy groups. An HSQC experiment revealed the hydrogen-bearing carbons of the three aromatic protons and the 11 methoxy groups (Table 3). In the HMBC spectrum the protons of nine methoxy groups correlated with one oxygen-bearing aromatic carbon, each indicating nine phenylmethyl ether functions in **11**. The remaining two methyl groups at δ 3.54 and 3.69 were linked to an ester carbonyl carbon each, as established by $^1H-^{13}C$ long-range correlations. The attachments of the phenylmethyl ether and methyl ester functions to the aromatic ring systems A−D were determined by $^1H-^{13}C$ long-range correlations (Figure 3).

As $^2J_{C-H}$, $^3J_{C-H}$, and $^4J_{C-H}$ long-range couplings cannot be distinguished in HMBC experiments, the exact position of each phenylmethyl ether group in **11** was established by NOE experiments. In the NOESY spectrum each of the aromatic protons showed cross-peaks to the protons of the adjacent methyl ether group and additionally in the case of H_A-6 and H_D-6‴ to the methyl ester group of rings A and D. NOESY correlations between neighboring methyl groups clearly indicated the position and sequence of each phenylmethyl ether function in the aromatic ring systems. The ether linkage between C-4″ of ring C and C-2‴ of ring D was deduced from the following considerations: The proton H_D-6‴ showed $^1H-^{13}C$ long-range correlations to three methyl-bearing phenolic carbons and to one without a methyl group. The NOESY experiment clearly established the 3‴-5‴ positions of the phenylmethyl ether groups in ring D, indicating that the phenolic carbon C-2‴was chemically modified before methylation.

In the HMBC spectrum H_C-6″ correlated with three phenolic carbons of which only the adjacent one was linked to a methoxy group. This observation led to the conclusion that one of the non-methoxylated phenolic carbons (C-3″) had to be assigned to the aromatic δ-lactone and the other (C-4″), in agreement with the molecular weight, to an ether bridge between rings C and D. The atropisomerism of the phenyl−phenyl bond of **11** ($[\alpha]^{26}_D = -33.9$) was established to be in the *S*-series by comparison of its CD curve with similar structures of known tannins.[13,15,18]

4,6-*O*-(*S*)-Isoterchebuloyl-D-glucose (**2**) was isolated as a tan amorphous powder. Its molecular formula of $C_{34}H_{22}O_{22}$ was established by HRFABMS. The ^1H and ^{13}C NMR spectra of **2** showed duplicated signals for the sugar and polyphenolic moieties, indicating an equilibrium mixture of α and β anomeric forms in solution (ratio: 4:3 of the α/β

Table 2. ^{1}H NMR and ^{13}C NMR Spectral Data of the HHDP and Isoterchebuloyl Units of **1** and **2** (acetone-d_6/D_2O = 9:1)

1						**2**		
isoterchebuloyl			HHDP			isoterchebuloyl		
pos	$^1H_{\alpha,\beta}$ [a]	^{13}C [a]	pos	$^1H_{\alpha,\beta}$ [a]	^{13}C [a]	pos	$^1H_{\alpha,\beta}$ [a]	^{13}C [a]
1		123.63,[b] 123.40b	1		114.59,[g] 114.46[g]	1		124.19, 123.96
2		116.50, 116.35	2		126.32, 126.11	2		116.12, 116.01
3		144.59	3	6.610, 6.615	107.42,[e] 107.36e	3		144.34
4		137.72, 137.63	4		145.16,[c] 145.15[c]	4		137.82, 137.78
5		145.21,[c] 145.17[d]	5		136.24, 136.15	5		145.15
6	6.79, 6.76	109.59, 109.51	6		144.41[h]	6	7.09	110.47, 110.40
7		167.45, 167.38	7		169.05, 168.95	7		167.75
1′		107.63,[e] 107.47[e]	1′		114.49,[g] 114.47[g]	1′		107.59, 107.53
2′		113.19, 113.14	2′		126.33	2′		113.51, 113.20
3′		136.66	3′	6.35, 6.37	106.93, 106.90	3′		137.43, 137.33
4′		139.58, 139.57	4′		145.26, 145.23[c]	4′		136.53
5′		147.86, 147.82	5′		136.27	5′		147.69, 147.66
6′		124.05,[b] 124.02[b]	6′		144.35,[h] 144.47[h]	6′		124.56, 124.52
7′		157.79, 157.75	7′		169.82	7′		157.78, 157.71
1″		113.61				1″		112.38
2″		112.83				2″		113.14, 112.85
3″		139.03,[f] 139.00[f]				3″		139.45, 139.44
4″		141.70, 141.64				4″		141.79, 141.72
5″		150.97, 150.91				5″		150.97, 150.91
6″	7.56, 7.57	112.55, 112.52				6″	7.540, 7.544	112.34
7″		160.0				7″		159.93
1‴		114.92, 114.77				1‴		115.12, 115.04
2‴		142.45				2‴		142.40
3‴		138.77,[f] 138.60[f]				3‴		138.90,[b] 138.89[b]
4‴		138.06,[f] 137.99[f]				4‴		138.60,[b] 138.44[b]
5‴		138.97[f,] 138.94[f]				5‴		138.92,[b] 138.91[b]
6‴	6.535, 6.530	107.55[e]				6‴	6.476, 6.482	107.39, 107.30
7‴		166.73, 166.67				7‴		166.82, 166.73

[a] ^{1}H NMR (500 MHz),^{13}C NMR 125.72 MHz (compound **1**) and 75.42 MHz (compound **2**). δ in ppm. Assignments based on GHSQC and HMBC spectra. ^{13}C NMR values without differentiation between α/β forms because of similar intensities of the ^{13}C NMR resonances. [b] $-h$ ^{13}C NMR values with same letters as well as values of positons 3′/4′, 3″/4″, 1′/2′, and 1″/2″ of the isoterchebuloyl unit may be interchanged.

Table 3. ^{1}H NMR (300 MHz) Spectral Data of **11** in Acetone-d_6 and MeOH-d_4 and ^{13}C NMR (75.42 MHz) Data of **11** in Acetone-d_6

pos	^{1}H (Me)[a]: acetone/MeOH	^{13}C (Me)[a]
1		126.26[b]
2		124.95[b]
3	(3.58/3.62)	151.85 (60.98)
4	(3.88/3.948)	146.64 (60.88)
5	(3.95/4.00)	153.66 (56.33)
6 (H_A)	7.46/7.54	110.17
7	(3.54/3.63)	166.75 (51.99)
1′		112.28
2′		116.19
3′		142.35
4′	(4.21/4.27)	146.24 (62.04)
5′	(3.66/3.71)	153.02 (61.40)
6′		133.50
7′		157.28
1″		113.57[c]
2″		114.18[c]
3″		140.54[d]
4″		140.00[d]
5″	(3.94/3.945)	153.61 (57.45)
6″ (H_C)	7.74/7.83	108.72
7″		159.01
1‴		118.73
2‴		145.56
3‴	(3.61/3.67)	146.54 (61.60)
4‴	(3.81/3.86)	147.34 (61.15)
5‴	(3.87/3.91)	150.53 (56.54)
6‴ (H_D)	7.21/7.28	109.15
7‴	(3.69/3.78)	165.83 (52.21)

[a] NMR data of methyl groups (Me) in parentheses. δ in ppm. Assignments based on NOESY, ROESY, NOE-difference, GHSQC, and HMBC spectra. [b] $-d$ ^{13}C NMR values with same letters may be interchanged.

forms in acetone-d_6/D_2O = 9:1). The sequence of the seven sugar protons in both anomeric sugar moieties and their directly bonded carbons was established by DQFCOSY and GHSQC, respectively (Table 1). Again, a $^4C_1$ conformation for the sugar parts of both isomers was established by the vicinal coupling constants of $J \approx 9$ Hz between the sugar protons H-2 and H-3, H-3 and H-4, and H-4 and H-5.

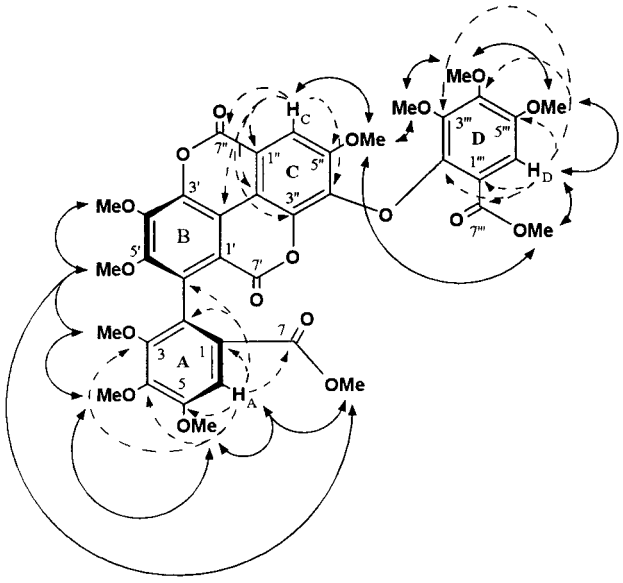


Figure 3. Nonamethylisoterchebulic acid dimethyl ester (**11**). Arrows show NOESY (↔) and HMBC correlations (→). ^{1}H−^{13}C long-range correlations between the methyl groups and their corresponding methoxy-bearing carbons are not shown.

The structure of the isoterchebuloyl moiety and its 4-O and 6-O linkage to the sugar core was elucidated by GHMQC experiments in a manner similar to that described for compound **1** (Table 2). The atropisomerism of the phenyl−phenyl bond of the isoterchebulic acid residue of **2** was deduced to be in the *S*-series from comparison of the optical rotation ($[\alpha]^{25}_D = -204$) and CD curve of **2** with corresponding data of the 4,6-*O*-(*S*)-isoterchebuloyl-D-glucose obtained by partial hydrolysis of **1**.

Compounds **1**−**8** were tested for their activities against the bacteria *Bacillus subtilis* and *Pseudomonas fluorescens*, the nematode *Caenorhabditis elegans*, and *Biomphalaria*

glabrata, one of the snail vectors of schistosomiasis, as well as the cancer cell lines Hep G2 (human hepatocellular carcinoma), MCF-7/S (N.I.H. human breast adenocarcinoma, pleural effusion), MDA-MB-231 (human breast adenocarcinoma), and 5637 cells (human primary bladder carcinoma). All compounds except **3** showed antimicrobial activities against *B. subtilis* in the microdilution assays:[20] minimal inhibition concentrations (MICs) of 8 μg/mL for compounds **1**, **2**, and **7**, 16 μg/mL for **8**, 32 μg/mL for **4**, and 64 μg/mL for **5** and **6**, compared to 2 μg/mL for the β-lactam antibiotic Cefotaxim. However, the observed activities originated from a bacteriostatic and not a bacteriocidal mode of action, as revealed by regrowth of bacteria treated with the MIC value of each compound on drug-free HNB agar plates. None of the compounds were active against *P. fluorescens* even in the highest concentration (1024 μg/mL). Only **1** exhibited moderate activity (++) at 100 μg/mL against the nematode *C. elegans*[21] and was molluscicidal against *B. glabrata* ($LC_{100} = 60$ μg/mL).[22] To determine the piscicidal property of **1**, this compound was tested against tilapia *Oreochromis mossambicus* at a concentration that was toxic to the snails (50 and 75 μg/mL).[23] At both concentrations compound **1** caused only 10% mortality. In the screening against the above-mentioned cancer cell lines only the compounds **3** and **8** were toxic against 5637 cells (human primary bladder carcinoma) with LC_{50} values of 84.66 ± 2.66 and 41.40 ± 3.34 μM, respectively.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi SMP-20 apparatus and are uncorrected. UV and CD spectra were obtained on a Hewlett-Packard 8452A diode array spectrometer and on a Jasco J-500A spectropolarimeter at 25 °C, respectively. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR spectra (KBr disks) were measured on a Perkin-Elmer FT-IR Paragon 1000 spectrometer.

NMR spectra were recorded on a Varian Unity Inova 500 or 300 MHz instrument. The ^{1}H and ^{13}C chemical shifts were referenced to solvent signals at $\delta_{H/C}$ 2.04/29.8 (acetone-d_6), 3.35/49.0 (methanol-d_4), 8.71/149.8 (pyridine-d_5), and 7.27/77.0 ($CDCl_3$) relative to TMS. All 1D (^{1}H, ^{13}C, NOE difference) and 2D NMR (COSY, DQFCOSY, NOESY, ROESY, GHSQC, GHMQC, G = gradient enhanced) measurements were performed using standard Varian pulse sequences. ^{1}H−^{13}C correlation spectra were recorded by GHSQC ($J_{C-H} = 140$ Hz) for the determination of proton-bearing carbons and GHMQC ($^nJ_{C-H}$ optimized for 1, 2, 3, 5, and 8 Hz; 4096 increments in F1) for multibond correlations (HMBC). NOESY and ROESY experiments were carried out with 0.5 and 1.0 s mixing time. ^{13}C NMR spectra were recorded at 125.72 and 75. 42 MHz with ~0.003 and ~0.004 ppm digital resolution (FT-size 128 K) to assign carbon shifts with two decimal places. Program for computer-generated carbon spectra: ACD-labs, version 2.0, 1996. Mass spectra were obtained on a Finnigan MAT 95 (FABMS, HRFABMS with NBA as matrix), a Finnigan MAT TSQ 700 (ESIMS), and a Varian MAT 311A (EIMS; 70 eV).

Open and low-pressure column chromatography was performed with polyamide SC60 (0.05−0.16 mm; Macherey&Nagel), Si gel G60 (40−63 μm; Merck), and Sephadex LH-20 (Fluka Biochemica). Analytical and semipreparative HPLC were performed on a Merck Hitachi 655A-12 liquid chromatograph equipped with a Shimadzu SPD-2A UV/vis detector and a Knauer HPLC Pump 64 equipped with an ABI Applied Biosystems 785A detector, respectively. Merck LiChroCART 125 × 4 mm cartridges (LiCrospher 100 RP18 and LiCrospher 100; 5 μm) were used for analytical and a Merck LiChroCART 250 × 10 mm cartridge (LiCrospher 100 RP18; 10 μm) as well as Bischoff HPLC 250 × 25 mm columns (LiCrospher 100 RP18, 10 μm and Spherisorb CN, 5 μm) for semipreparative separations. Thin-layer chromatography was carried out on precoated Si gel 60 F_{254} plates (Merck), and spots were detected by UV illumination.

Plant Material. The bark of *T. macroptera* (Combretaceae) was collected at Wakwa, Cameroon, in 1995 and identified by Dr. S. Yonkeu, Chief of the herbarium at the Institute de Recherche Zoo Veterinaire, Wakwa, Cameroon. A voucher specimen (no. 47542 HNC) is located at the Cameroon National Herbarium, Ngaoundere, Cameroon.

Extraction and Isolation. The air-dried powdered bark of *T. macroptera* was extracted and treated as previously described.[11] Sephadex LH-20 chromatography of the EtOAc extract (40 g) with MeOH as eluant afforded seven fractions, A_1−A_7. HPLC purification [5 mL/min, 250 × 25 mm, CH_3CN−H_2O (32:68) + 0.01% TFA, UV 280 nm] of an aliquot (1.4 g) of fraction A_6 (8.1 g) on RP 18 afforded compound **1** (1.2 g). The *n*-BuOH extract (106 g) was partitioned between $CHCl_3$, MeOH, and H_2O (1.5:1:1). The aqueous layer was extracted six times with the organic phase and concentrated under reduced pressure to give 70 g of a brown crystalline residue. A part of this residue (36 g) was subjected to a polyamide SC60 column (200 g) and eluted with MeOH−H_2O (2:8, 5:5, 10:0) → MeOH−EtOH (5:5, 0:10) → EtOH−acetone−DMF−*n*-PrOH (5:1:1:3). Fractions were monitored by analytical HPLC (1 mL/min, RP 18, CH_3CN−H_2O (10:90) and (15:85) + 0.07% TFA, UV 254 nm), and fractions with the same HPLC pattern were combined to yield 11 fractions, B_1−B_{11}, altogether. Comparison of the retention times and ^{1}H NMR spectra of fractions B_{10} and B_{11} (15.7 g in total) revealed that **1** was the only compound in these fractions.

Fraction B_3 was dissolved in 10 mL of MeOH−H_2O (1:1) and centrifuged. The evaporated supernatant (300 mg) was purified by HPLC on RP18 [5 mL/min, 250 × 10 mm, H_2O + 0.05% TFA, UV 254 nm] to afford compound **3** (216 mg). An aliquot (500 mg) of fraction B_4 (1 g) was applied to HPLC on RP18 [5 mL/min, 250 × 10 mm, CH_3CN−H_2O (2:98 → 7:93) + 0.05% TFA, UV 254 nm] to give compound **6** (35 mg). An aliquot (500 mg) of fraction B_9 (5.6 g) was purified by HPLC on RP18 [5 mL/min, 250 × 10 mm, CH_3CN−H_2O (10:90 → 13:87) + 0.05% TFA, UV 254 nm] to yield compounds **4** (58 mg) and **5** (23 mg).

Compounds **2**, **7**, and **8** found in fraction B_9 (4 g) could not be separated by HPLC on RP18. Therefore, B_9 was subjected to Sephadex LH-20 chromatography with MeOH−H_2O (9:1) as eluant to afford nine fractions, C_1−C_9. HPLC purification [5 mL/min, 250 × 10 mm, CH_3CN−H_2O (7.5:92.5) + 0.05% TFA, UV 254 nm] of fraction C_4 (347 mg) gave compound **2** (84 mg) and a mixture of compounds **7** and **8**. HPLC on CN [5 mL/min, 250 × 25 mm, CH_3CN−H_2O (0.4:99.6) + 0.075% TFA, UV 254 nm] of this mixture yielded pure compounds **7** (28 mg) and **8** (40 mg).

Isoterchebulin (**1**): tan amorphous powder; mp (H_2O) 240−245 °C (decomp); $[\alpha]^{25}_D$ −169°(*c* 0.465, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.84), 258 (4.82), 378 (4.13) nm; CD (*c* 2.5 × 10^{-5}, MeOH) θ (nm) −4.16 × 10^4 (215), +5.68 × 10^4 (237), −2.24 × 10^4 (295), −0.56 × 10^4 (372); IR (KBr) ν_{max} 3416, 1733, 1610, 1517, 1458, 1360, 1312, 1180, 1059, 965, 877, 836, 770, 746 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; negative ESIMS and FABMS *m*/*z* 1083 $[M - H]^-$; HRFABMS *m*/*z* 1083.0588 $[M - H]^-$ (calcd for $C_{48}H_{27}O_{30}$ requires 1083.0576).

Partial Hydrolysis of 1. A solution of **1** (80 mg) in 1 N H_2SO_4 (5 mL) was heated under reflux for 6 h. After cooling, the reaction mixture was neutralized with Na_2CO_3 and the solvent removed in vacuo. The residue was dissolved in MeOH−H_2O (1:1; 20 mL) and filtered. The filtrate was concentrated under reduced pressure and applied to HPLC on RP18 [5 mL/min, 250 × 10 mm, MeOH−H_2O (5:95 → 100:0 in 30 min) + 0.1% TFA, UV 254 nm] to give the starting material (17 mg), ellagic acid (8 mg), and a hydrolysate identical to compound **2**: $[\alpha]^{25}_D$ −196° (*c* 0.5, MeOH); ^{1}H and ^{13}C NMR identical to **2** (Tables 1 and 2), negative ESIMS *m*/*z* 781 $[M - H]^-$. Ellagic acid: ^{1}H NMR (pyridine-d_5, 300 MHz) δ 8.2; ^{13}C NMR (pyridine-d_5, 75.42 MHz) δ 160.9, 142.1, 137.8, 113.8, 111.8, 108.7; EIMS *m*/*z* 302 $[M]^+$ (61), 44 (100), 28 (55).

Sugar Analysis of 1. A solution of **1** (150 mg) in 3 N TFA (8 mL) was heated under reflux for 24 h. After cooling, the

precipitate was removed by filtration and the filtrate evaporated to dryness. The residue was subjected to a polyamide SC60 column (5 g, 0.1% TFA) followed by chromatography on Si gel [10 g, $CHCl_3$−MeOH−H_2O (60:35:5)]. Final purification was achieved by chromatography on NH_2 phase (solid-phase extraction cartridge Adsorbex, Merck) using CH_3CN as eluant. The resulting D-glucose (11 mg) was identified by TLC and determination of its optical rotation, $[\alpha]^{25}_D$ +59 (*c* 0.5, H_2O), in comparison with an authentic sample (Merck).

Methylation of 1. A mixture of **1** (500 mg), dimethyl sulfate (2 mL), and anhydrous K_2CO_3 (2.8 g) in dry acetone (25 mL) was heated under reflux for 4 h. After cooling, the precipitate was removed by filtration. The resulting filtrate was acidified with formic acid (pH 4) and concentrated under reduced pressure. The residue was chromatographed on Si gel (35 g) with petroleum ether−EtOAc (60:40 → 35:65) to afford the hexadecamethyl ether **9** (279 mg) as a yellow crystalline powder; positive ESIMS *m/z* 1309 $[M + H]^+$, 1331 $[M + Na]^+$, 1347 $[M + K]^+$; ^1H NMR (acetone-d_6, 500 MHz) δ 7.71, 7.02 (2H in total, each s, aromatic H), 6.85, 6.82, 6.80, 6.79, 6.77, 6.67 (3H in total, each s, aromatic H), 4.30, 4.29, 4.00, 3.98, 3.94, 3.93, 3.89, 3.88 (×2), 3.87, 3.86, 3.84, 3.83, 3.82, 3.79, 3.78, 3.77 (×2), 3.73, 3.71, 3.70, 3.66, 3.65, 3.61, 3.60, 3.42, 3.32 (each s, total 16 CH_3), 5.54 (t, J = 9.5 Hz), 5.40 (m), 5.04 (dd, J = 3.7, 9.7 Hz), 4.99 (d, J = 3.7 Hz), 4.89 (t, J = 9.8 Hz), 4.81 (m), 4.60 (dd, J = 9.0, 12.0 Hz), 4.59 (dd, J = 8.8, 12.0 Hz), 3.02 (bd, J = 12.0 Hz), 3.00 (bd, J = 12.0 Hz) (12H in total, α/β sugar protons).

Alkaline Methanolysis of 9. A solution of **9** (110 mg) in dry methanol (50 mL) was treated dropwise with 5% sodium methoxide to a final sodium methoxide concentration of 0.04% in the reaction mixture. After 16 h at room temperature the reaction mixture was neutralized with acetic acid, filtered, and concentrated under reduced pressure. Chromatography of the residue on Si gel (20 g) with petroleum ether−EtOAc (50:50 → 35:65) yielded the colorless oily dimethyl-(*S*)-hexamethoxydiphenoate (**10**) (17 mg) and nonamethylisoterchebulic acid dimethyl ester (**11**) (14 mg) as a pale yellow powder. Compound **10**: $[\alpha]^{25}_D$ −25° (*c* 1.69, $CHCl_3$); CD (*c* 8.89 × 10^{-5}, CH_3CN) θ (nm) −4.33 × 10^4 (248), +0.79 × 10^4 (308); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.37 (2H, s, aromatic H), 3.94, 3.93, 3.60 (×2), (each s, 8 CH_3); positive ESIMS *m/z* 451 $[M + H]^+$. Compound **11**: $[\alpha]^{26}_D$ −33.9° (*c* 0.36, $CHCl_3$); UV (CH_3CN) λ_{max} (log ϵ) 214 (4.72), 254 (4.79), 370 (4.21) nm; CD (*c* 5.13 × 10^{-5}, CH_3CN) θ (nm) −4.37 × 10^4 (242), +0.21 × 10^4 (257), −1.57 × 10^4 (270), +1.33 × 10^4 (312); ^1H and ^{13}C NMR, see Table 3; positive ESIMS *m/z* 815 $[M + Na]^+$.

4,6-*O*-(*S*)-Isoterchebuloyl-D-glucose (2): tan amorphous powder; mp (H_2O) 212−215 °C (decomp); $[\alpha]^{25}_D$ −204° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.70), 262 (4.70), 378 (4.10) nm; CD (*c* 2.5 × 10^{-5}, MeOH) θ (nm) −7.66 × 10^4 (218), +5.83 × 10^4 (250), −0.80 × 10^4 (298), −1.20 × 10^4 (372); IR (KBr) ν_{max} 3413, 1723, 1608, 1521, 1459, 1435, 1362, 1178, 1059, 1017, 963, 881, 837, 771, 723 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; negative ESIMS and FABMS *m/z* 781 $[M - H]^-$; HRFABMS *m/z* 781.0554 $[M - H]^-$ (calcd for $C_{34}H_{21}O_{22}$ requires 781.0516).

Antimicrobial Activity. Samples in serial dilutions [starting concentration 1024 μg/mL, dilution factor $1/2^n$, two replicates for each concentration] were tested in microdilution assays according to established protocols[20] using *B. subtilis* and *P. fluorescens* as test organisms. The β-lactam antibiotic Cefotaxim was used as a positive control (MIC 2 μg/mL).

Anthelminthic Activity. The bioassay against *C. elegans* was performed according to Simpkin and Coles.[21] Compounds were tested at 50 and 100 μg/mL (n = 60, in two replicates for each concentration), and biotests were evaluated by assessing the increase in number of worms and their movement.[11] The anthelminthic Pyrantelembonate was used as a positive control with strong activity (+++) at 50 μg/mL.

Molluscicidal Activity. Molluscicidal properties of the compounds against *B. glabrata* were examined using a rapid screening method.[22] Samples were tested at 100 μg/mL (n = 6, in three replicates). In case of toxicity LC_{100} were determined by serial dilutions (80, 60, 40, 20, 10 μg/mL) of the active compounds (n = 6, in three replicates for each concentration). Bayluscid was used as positive control (LC_{100} 5 μg/mL).

Piscicidal Activity. Compound **1** was tested at 50 and 75 μg/mL (n = 30, in three replicates for each concentration) against *O. mossambicus* using the standard protocol according to Balza.[23]

Cytotoxic Assay. Compounds **1**−**8** were screened against Hep G2 (human hepatocellular carcinoma), MCF-7/S[24] (N.I.H. human breast adenocarcinoma, pleural effusion), MDA-MB-231 (human breast adenocarcinoma), and 5637 cells (human primary bladder carcinoma) using the method according to Setzer.[25]

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Supporting Information Available: Structures of **1**−**8**, part of the GHMBC spectrum of **1**, derivatization and degradation scheme of **1** and CD spectrum of **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) von Maydell, H.-J. *Trees and Shrubs of the Sahel*; Verlag Joseph Margraf: Weikersheim, 1990; pp 63−76 and 393.
(2) le Grand, A.; Wondergem, P. A.; Verpoorte, R.; Pousset, J. L. *J. Ethnopharmacol*. **1988**, *22*, 25−31.
(3) Silva, O.; Ferreira, E.; Vaz Pato, M.; Gomes, E. *Int. J. Pharmacogn*. **1997**, *35*, 323−325.
(4) Sandberg, F.; Cronlund, A. *J. Ethnopharmacol*. **1982**, *5*, 187−204.
(5) Nongonierma, R.; Proliac, A.; Raynaud, J. *Pharmazie* **1987**, *42*, 871−872.
(6) Nongonierma, R.; Proliac, A.; Raynaud, J. *Pharmazie* **1988**, *43*, 293.
(7) Nongonierma, R.; Proliac, A.; Raynaud, J. *Pharm. Acta Helv*. **1990**, *65* (8), 233−235.
(8) Noguetra, L.; de Almeida e Silva, L.; Correira Alves, A. *Garcia Orta* **1962**, *10*, 501−509; *Chem. Abstr*. **1964**, *61*, 12326.
(9) Silva, O.; Ferreira, E.; Romao, N.; Franco, S.; Porem, L.; Esmeraldo, N.; Vaz Pato, M.; Gomes, E. T. In *Book of Abstracts of the Joint Meeting of the American Society of Pharmacognosy*; GA, Phytochemical Society of Europe and AFERP: Amsterdam, 1999; abstract 429.
(10) Idemudia, O. G. *Phytochemistry* **1970**, *9*, 2401−2402.
(11) Conrad, J.; Vogler, B.; Klaiber, I.; Roos, G.; Walter, U.; Kraus, W. *Phytochemistry* **1998**, *48* (4), 647−650.
(12) Conrad, J.; Vogler, B.; Klaiber, I.; Reeb, S.; Guse, J.-H.; Roos, G.; Kraus, W. *Nat. Prod. Lett*. **2000**, *15*, 35−43.
(13) Jossang, A.; Pousset, J. L.; Bodo, B. *J. Nat. Prod*. **1994**, *57*, 732−737, and references therein.
(14) Doig, A. J.; Williams, D. H.; Oelrichs, P. B.; Bacynskyi, L. *J. Chem. Soc., Perkin Trans. 1* **1990**, 2317−2321, and references therein.
(15) Tanaka, T.; Nonaka, G. I.; Nishioka, I. *Chem. Pharm. Bull*. **1986**, *34*, 1039−1049.
(16) Tanaka, T.; Nonaka, G. I.; Nishioka, I. *Chem. Pharm. Bull*. **1986**, *34*, 650−655.
(17) Tanaka, T.; Nonaka, G. I.; Nishioka, I. *Chem. Pharm. Bull*. **1986**, *34*, 656−663.
(18) Lin, T. C.; Nonaka, G. I.; Nishioka, I.; Ho, F. C. *Chem. Pharm. Bull*. **1990**, *38*, 3004−3008.
(19) Li, X.-C.; Elsohly, H. N.; Hufford, C. D.; Clark, A. M. *Magn. Reson. Chem*. **1999**, *37*, 856−859.
(20) (a) Vanden Berge, D. A.; Vlietinck, A. J. In *Methods in Plant Biochemistry*; Dey, P. M., Harborne, J., Eds.; Academic Press: London, 1991; Vol. 6, pp 52−57. (b) "Arbeitsausschuss chemotherapeutischer Untersuchungsmethoden" des Normenausschusses Medizin (NAMed); DIN 58940, part 8. In *Medizinische Mikrobiologie und Immunologie*; DIN pocket book 222; Beuth Verlag: Berlin, Köln, 1991.
(21) Simpkin, K. G.; Coles, G. C. *J. Chem. Technol. Biotechnol*. **1981**, *31*, 66−69.
(22) (a) Moser, D.; Klaiber, I.; Vogler, B.; Kraus, W. *Pestic. Sci*. **1999**, *55*, 336−339. (b) Nakanishi, K.; Kubo, I. *Isr. J. Chem*. **1977**, *16*, 28−31. (c) Marston, K.; Hostettmann, K. In *Methods in Plant Biochemistry*; Dey, P. M., Harborne, J., Eds.; Academic Press: London, 1991; Vol. 6, pp 153−178.
(23) Balza, F.; Abranowski, Z.; Towers, G. H. N.; Wiryachitra, P. *Phytochemistry* **1989**, *23*, 1827−1830.
(24) Osborne, C. K.; Hobbs, K.; Trent, J. M. *Breast Cancer Res. Treat*. **1987**, *9* (2), 111−121.
(25) Setzer, M. C.; Setzer, W. N.; Jackes, B. R.; Gentry, G. A.; Moriarity, D. M. *Pharm. Biol*. **2000**, in press.

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